

# 13th Meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): Alternative Testing Methodologies for Ecotoxicity

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There is growing public pressure to minimize the use of vertebrates in ecotoxicity testing; therefore, effective alternatives to toxicity tests causing suffering are being sought. This report discusses alternatives and differs in some respects from the reports of the other three groups because the primary concern is with harmful effects of chemicals at the level of population and above rather than with harmful effects upon individuals. It is concluded that progress toward the objective of minimizing testing that causes suffering would be served by the following initiatives—a clearer definition of goals and strategies when undertaking testing procedures; development of alternative assays, including *in vitro* test systems, that are based on new technology; development of nondestructive assays for vertebrates (e.g., biomarkers) that do not cause suffering; selection of most appropriate species, strains, and developmental stages for testing procedures (but no additional species for basic testing); better integrated and more flexible testing procedures incorporating biomarker responses, ecophysiological concepts, and ecological end points (progress in this direction depends upon expert judgment). In general, testing procedures could be made more realistic, taking into account problems with mixtures, and with volatile or insoluble chemicals. — *Environ Health Perspect* 106(Suppl 2):441–451 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-2/441-451/walker/abstract.html>

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## Introduction— Definition of Aims

Concerns over the use of animals in toxicological studies are gaining increasing political force. There is the paradox of growing public demand for greater safety regarding the effects of chemicals on human health

and the environment, which is in conflict with the increasing pressure for fewer or even no animal experiments. The rationale of alternative testing is to minimize, as far as possible, the use of vertebrates in testing

procedures. When vertebrates are used, the numbers and the suffering involved should be decreased as far as possible without affecting the validity of the testing.

Three major thrusts towards alternative testing are considered here.

- The use of lower organism as surrogates for vertebrates in toxicity testing (1,2). This concept is considered in the sections on “Present Testing Requirements” and “Alternative Methods Already Developed.” A detailed discussion of the correlation of toxicity testing between fish and bacteria is considered in detail by Kaiser (3).
- Nondestructive techniques in vertebrate testing such as the use of blood, skin, or excreta, quantitative structure–activity relationships (QSARs), and the development of cell cultures. These concepts are considered in the sections on “Alternative Methods Already Developed” and “Future Developments.”
- Methodologies that indicate environmental health (for example, the River Invertebrate Prediction and Classification System [RIVPACS] and the use of biomarkers in field studies) can indicate whether testing is necessary. This concept is discussed in the section on “Methodology to Assess Environmental Health (Quality) to Establish Whether Testing Is Needed.” The strategies of using biomarkers are considered in more detail by Walker (4).

In general, ecotoxicologists are concerned more with the health of populations and communities rather than that of the individual; therefore, the thrust of this section differs from other joint reports in SCOMSEC 13. The complexity of the task is enormous because there are literally millions of species and all these species interact with other species. The selection of sentinel species is considered in detail by Sheffield and Kendall (2). The complexity increases as we move from vertebrates (numbered in thousands of species), which in the past have largely been the sentinel species of choice, to invertebrates (numbered in millions). The use of invertebrates in alternative testings of environmental chemicals is discussed by Lagadic and Caquet (1).

The ecotoxicological section of this report is divided into four main sections:

- Description of standard toxicity testing, discussing preregistration tests and additional tests that they may trigger.

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Abbreviations used: 3Rs, refinement, reduction, and replacement (of animal use); ATP, adenosine triphosphate; FAO, Food and Agriculture Organization, United Nations; GLP, Good Laboratory Practices; IBI, biotic integrity; LD<sub>50</sub>, lethal dose 50; ODS, ozone-depleting substances; OECD, Organisation for Economic Co-operation and Development; QSAR, quantitative structure–activity relationship; PAH, polycyclic aromatic hydrocarbon; PCBs, polychlorinated biphenyls; PCDD, polychlorinated dibenzo-*p*-dioxin; PCDF, polychlorinated dibenzofurans; RAPD, randomly amplified polymorphic DNA/RIVPACS; SLRLT, sex-linked recessive lethal test; SMART, somatic mutation and recombination test; WHO, World Health Organization.

- Methodology to assess environmental health and establish whether additional testing is required.
- Alternative methods that have already been developed. These range from those involving cell cultures, individuals, and populations, to communities and ecosystems. Mesocosms also have been used with a variety of end points.
- Finally, the future development of the field is considered, looking at such subjects as the increasing use of cell cultures, refinement of nondestructive testing, and the extrapolation between levels of biological organization.

## Present Testing Requirements

Environmental risk assessment differs from human risk assessment in two important respects. First, a very small number of surrogates in toxicity testing represent a huge number of wild species, making extrapolation of data particularly difficult. Second, environmental exposures of wild species to chemicals are very hard to estimate with any degree of accuracy, especially in the terrestrial environment. Consequently, large safety factors are used in environmental risk assessment.

## Chemical Assessment

Regulations differentiate between existing and new chemicals (5). Whereas the U.S. regulation for new substances requires data primarily on the identity of substances and the Japanese regulation focuses on bioaccumulation and biodegradation, the European directive fixes a full test program on dangerous substances. The number of tests to be conducted depends on the amount of the substance placed on the market.

For example, under the European directive, tests to be conducted in the field of ecotoxicological effects include:

- < 100 kg/year; no ecotoxicological data
- < 1 time/year; biodegradability only
- > 1 time/year; fish, *Daphnia*, algae, bacteria, degradation, absorption/desorption
- > 100 times/year; additional tests, e.g., on chronic effects including vertebrates and tests on soil organisms

Based on the test results and the exposure estimation, risk assessments for all dangerous new substances must be conducted. If the risk assessment shows that the substance is of concern and the data is not sufficient, additional tests must be conducted.

Consequently, the most important issue regarding stringent test requirements

is alternatives for the acute and prolonged fish test and the fish bioaccumulation test.

## Pesticides

A questionnaire to Organisation for Economic Co-operation and Development (OECD) member countries regarding environmental risk assessments and regulatory decisions revealed that all 13 responding countries assess risk to species and populations. Most countries include single habitats and ecosystems. Testing is requested on active ingredients and on commercial formulation by all countries, and all countries except one also require testing on metabolites. For aquatic toxicity, all countries

require acute data on *Daphnia* and fish; most require additional testing on algae, sediment organisms, and other aquatic invertebrates. Chronic data on *Daphnia* and fish are requested by most countries (Table 1). Furthermore, most countries use data from microcosms if available, and some request testing with microcosms, mesocosms, and field experiments on a case-by-case basis.

Requirements in terrestrial testing include birds and other wildlife species (6), honey bees, and mammals in most countries; some countries require testing on soil microorganisms, earthworms, plants, other soil organisms, and beneficial arthropods (Table 2).

**Table 1.** Acute aquatic toxicity information required for commercial products and/or metabolites.

	Acute effects												
	A	B	CAN	CH	D	DK	FIN	J	N	NL	S	UK	USA
Algae		X	X	X		X	Fin1		X <sup>N1</sup>	X <sup>NL1</sup>	X	X <sup>UK1</sup>	
<i>Daphnia</i>	X	X	X	X	X	X	Fin1	X	X <sup>N1</sup>	X <sup>NL1</sup>	X	X <sup>UK1</sup>	X
Fish	X	X	X	X	X	X	Fin1	X	X <sup>N1</sup>	X <sup>NL1</sup>	X	X <sup>UK1</sup>	X
Sediment organisms		X <sup>B1</sup>	X <sup>CAN1</sup>	X <sup>CH1</sup>		(X)	Fin1					X <sup>UK1</sup>	X <sup>USA2</sup>
Other			X <sup>CAN2</sup>	X <sup>CH1</sup>		(X)	Fin1				X <sup>S1</sup>	X <sup>UK1,2</sup>	X <sup>USA1</sup>

B1, if this is the compartment of concern; CAN1, case by case; CAN2, aquatic vascular plants, marine organisms; CH1, if warranted by the substance properties and the envisaged use pattern; DK, on the active ingredient. Data on commercial products and/or metabolites are used if available; (X), if available; FIN, the need is evaluated case by case; N1, data required for metabolites > 10% not for commercial products; NL1, data required for metabolites > 10% in a water sediment study, not for commercial products; S1, studies on aquatic vascular plants required for herbicides; UK1, tests are not automatically required: only when use pattern, formulation type and initial risk assessment require product tests they are required; UK2, other aquatic invertebrates; USA1, estuarine organisms; USA2, new requirements drafted for regulatory purposes. A, Austria; B, Belgium; CAN, Canada; CH, Switzerland; D, Germany; DK, Denmark; FIN, Finland; J, Japan; N, Norway; NL, The Netherlands; S, Sweden; UK, United Kingdom; US, United States.

**Table 2.** Acute terrestrial toxicity information required for commercial products and metabolites.

	Acute effects												
	A	B	CAN	CH	D	DK	FIN	J	N	NL	S	UK	USA
Soil micro-organisms		X <sup>B1</sup>		X		X	Fin1						
Earthworms	X	X <sup>B1</sup>		X	X <sup>D1,2</sup>	X	Fin1						USA <sup>1</sup>
Other soil organisms	X			X			Fin1						USA <sup>1</sup>
Plants			X	X			Fin1	X		X			
Other beneficial arthropods	X	X <sup>B1</sup>	X	X	X <sup>D1,3</sup>		Fin1	X					USA <sup>1</sup>
Birds	X <sup>A1</sup>	X <sup>B1</sup>	X	X	X <sup>D2</sup>	X	Fin1				X <sup>S1</sup>	X <sup>S1</sup>	X
Mammals	X		X	X	X <sup>D1,2</sup>	X	Fin1						X
Honeybees	X	X <sup>B1</sup>	X	X	X <sup>D1</sup>	X	Fin1	X	X		X <sup>S2</sup>	X <sup>S2</sup>	X

A1, if 10 < toxicity exposure ratio of a.i. < 100, or LD<sub>50rat</sub> of preparation < LD<sub>50a.i.</sub>; B1, required depending on the values of TER of the a.i.; D1, for commercial products; D2, for active ingredients or metabolites; D3, dependent on species and available tests; DK, on the active ingredients. Data on commercial products and/or metabolites are used if available; FIN1, before EU-membership such information was not required regularly. The need for information on terrestrial effects of commercial products and metabolites was evaluated case by case making expert judgement; S1, required for commercial products formulated as granules, seed dressings, etc. S2, required for commercial products intended for use on plants during the flowering period. USA1, although they are rarely imposed, the United States has test guidelines for earthworms, other soil organisms, and other beneficial arthropods. A, Austria; B, Belgium; CAN, Canada; CH, Switzerland; D, Germany; DK, Denmark; FIN, Finland; J, Japan; N, Norway; NL, The Netherlands; S, Sweden; UK, United Kingdom; USA, United States of America.

### Regulatory Practice in Dealing with Uncertainty

Depending on the size of ecotoxicological datasets and how representative they are for realistic environmental situations, application factors are commonly used and included in testing schemes. Irrespective of strict testing requirements, this practice involves testing on more and more species, and attempts to establish environmental effect levels have resulted in many more vertebrate experiments than are required by law. Many pesticides have been repeatedly tested with dozens of fish species in this context.

Considering the increasing reliability of QSARs, the mechanistic approach as an alternative to dealing with uncertainty could lead to a significant reduction in vertebrate testing. Availability of test results could also decrease numbers of animals used.

### Methodology to Assess Environmental Health (Quality) to Establish Whether Testing Is Needed

Deciding whether studies should be undertaken to determine the extent of environmental damage is one of the most critical questions in the environmental field in general, not just to those concerned with alternative testing. The development and refinement of analytical chemistry techniques have allowed determination of very low levels of environmental pollutants. The desire to know what levels of pollutants exist have generated a vast amount of data on environmental levels of organic and inorganic chemicals. Regrettably, very little of these data can be interpreted in biological terms. The harmful effects, if any, of these chemicals upon living organisms remain largely unknown. Experimental work to determine these effects is hampered not only by the number of chemicals and species involved, but also by the fact that environmental chemicals almost always occur as mixtures, and the fact that we are interested in effects on populations, communities, and ecosystems as well as individuals.

Two approaches that have been employed to address these problems are the use of biomarkers and direct observations on community structure.

Definitions of biomarkers have varied considerably and have frequently been so broad as to include any biological change. Here, we use the definition put forward by Peakall and Walker (7): "any biological

response to an environmental chemical at the individual level or below which gives a measure of exposure and sometimes also of toxic effect." Biochemical, physiological, immunological, histological, morphological, and behavioral changes are included in this definition.

Biomarkers have an advantage over chemical analysis in that they can demonstrate whether an organism is meaningfully exposed. Analytical chemistry is now so sophisticated that in almost all samples environmental pollutants can be detected, but the physiological significance is rarely known. With biomarkers it is possible to determine if the biochemistry and physiology of the organism is significantly different from normal. If they are, the organism can be considered to be meaningfully exposed, and equally important, if they are not significantly different, the organism can be considered not meaningfully exposed even though the chemicals can be detected. The ability to determine if an organism is meaningfully exposed is important in the decision-making process as to whether further studies should be undertaken. The following criteria must be met before the concept of meaningful exposure can be used. These are

- Control data and the degree of normal variation must be available for each biomarker. This is a good deal more complex than using biochemical levels for the diagnosis of human health because of the diversity of species and naturally occurring fluctuations involved. Obviously, it is impossible to have data on all species; the question of the selection of sentinel species is critical. This issue is discussed by Sheffield and Kendall (2).
- Good biomarkers must be developed to indicate the health of the major functions of the organism (growth, reproduction) and to be able to assess the impact of the major classes of chemicals of concern. Although we have not reached this point, progress so far is encouraging.

The biomarker approach can address the problem of mixtures by showing whether an organism in a specific environment shows departures from normality that may be regarded as harmful. Only if deviations from normality are found, and only if these deviations are serious, are additional studies warranted. Some adaptive changes such as the induction of mixed-function oxidases may be considered acceptable even though they demonstrate exposure. It is necessary to determine which chemical(s) are causing

changes only at the stage that changes are considered deleterious.

A direct method of determining ecosystem health is by assessing whether the community structure is disturbed. Although the methods involve collection of organisms for taxonomic identification, this process has significant adverse effects on the ecosystem only in exceptional circumstances.

One of best-validated systems, the RIVPACS, has been promoted for the assessment of the biological quality of rivers in the United Kingdom (8). The system is used to generate site-specific predictions of the principal macro invertebrate fauna expected in the absence of major environmental stresses. The fauna predicted can then be compared with the fauna observed. Differences between the two indicate the presence of environmental stresses requiring investigation. Another use of RIVPACS is to establish a national classification of sites that not only provides a basis for pollution control, but also indicates sites of high biological quality. There are currently some 9000 sites in the United Kingdom used to assess biological quality in this way.

A related index that is receiving considerable attention in the United States for assessing contamination of aquatic contamination is the Invertebrate Community Index, a system that derives a quantitative index of aquatic systems based on aquatic macro invertebrate community structure and function.

Another measure of effects of contaminants on aquatic communities is the Index of Biotic Integrity (IBI) (9). This index assesses fishes and the physical characteristics of the water to derive a quantitative index of aquatic community integrity. It can be used to monitor general aquatic health and to assess impact of contaminants following chemical spills. IBI is used in several states of the United States as a regulatory mechanism for aquatic system health. The problem with adopting IBI for wider regulatory use is the geographical variability of the aquatic systems.

Less advanced are terrestrial systems, but studies on soils using macro invertebrates are currently being developed.

As no environment can now be considered pristine we consider that one of the most critical aspects of ecotoxicology is the determination of whether the health of the system is acceptable so that resources can be allocated for additional studies and remedial action as required.

## Alternative Methods Already Developed

A survey of scientific literature between 1992 and spring 1996 reveals that the use of invertebrate alternatives is marginal and primarily involves developmental toxicity and genotoxicity testing (Table 3).

### Upon Individuals

The use of cell systems to measure responses to xenobiotics has been widely investigated in the quest for alternative methods in toxicity testing. However, the development of such methods in the context of environmental risk assessment has been relatively recent and limited in scale (4). Two rather different approaches have been followed. On one hand, fish hepatocytes have been employed to measure increases in stress proteins, cytochrome P4501A1, DNA adducts, and vitellogenin levels in response to a range of organic pollutants (10). In contrast, trans-fected mammalian cell lines have been developed; these have been used to obtain an integrated measurement of the interaction of mixtures of polyhalogenated hydrocarbons (polychlorinated biphenyls [PCBs], polychlorinated dibenzo-*p*-dioxin [PCDDs], polychlorinated dibenzofurans [PCDFs]) with the Ah receptor, an interaction that is associated with a number of toxic responses under *in vivo* conditions. This method depends upon the incorporation of a "reporter" gene into the DNA of the hepatocytes. When chemicals bind to the Ah receptor in the cytosol, a signal is sent to the DNA and through the mediation of the reporter gene leads to the synthesis of the

enzyme luciferase. Luciferase then generates photons, which can be measured in the assay system.

In the examples given, the measured responses are related to toxicity, an attractive feature of this type of alternative assay. They can provide a direct measurement of the elicitation of a toxic response to chemicals acting singly or in combination. The difficulty comes in relating the responses that occur *in vitro* to effects that would be produced *in vivo* given the same dosing regimen. Establishing quantitative relationships between *in vitro* and *in vivo* responses is not straightforward.

**Microorganisms.** A number of bacteria, yeasts, and other microorganisms have been used to assess the effects of chemicals and complex mixtures. Biological waste treatment systems rely heavily on viable microbial assemblages to degrade the waste constituents. In the testing of individual organisms, typical end points include growth, spore germination, and, in the case of luminescent organisms, change in light emission. One widely used, commercially available organism and test system uses the marine bacterium *Vibrio fischeri* (formerly *Photobacterium phosphoreum*). This test has recently been adopted as a standard test in several countries and is finding increasing acceptance. At this time, more than 1500 chemicals have been tested in this system and the data are readily available (11).

The most frequently used vertebrates in ecotoxicity testing for legal purposes are fish. Acute toxicity to fish is required in the

lowest tier testing. Although the United States accepts QSAR estimates in the premarketing stage of assessment, the European Union requires experimental testing for all chemicals. Categories of chemicals requiring registration may need information on prolonged fish testing; depending on results, go to further tiers with early life-stage or even full life-cycle testing. The major use of animals, however, is in acute and chronic testing.

**In Vitro Testing.** Investigations with different life stages of fish, including fertilized eggs, have a long tradition originally targeting identification of the most sensitive developmental phase (12–14). The overall outcome has been that early life stages are more sensitive to most chemicals than adult fish.

**Testing Acute Toxicity with Zebrafish Eggs.** With the objective of replacing the acute fish test, a test with fertilized eggs of zebrafish has been developed in a concerted program (15). Because the embryos will not hatch during the test period, it is classified as a nonanimal test.

Exposure in the test is for 48 hr. Assessed end points are coagulation, development of blastula, gastrulation, termination of gastrulation, development of somites, movements, extension of the tail, development of eyes, heartbeat, circulation, heart rate, pigmentation, and edema. Endpoints comparable with lethality in acute toxicity tests are no completion of gastrulation (12 hr); no somites (16 hr); no heartbeat (48 hr); no movement (48 hr); coagulated eggs. The other end points give further insight for a more detailed assessment of the effects potential of the test substances.

**Validation Comparison with Results from Fish Acute Toxicity Tests.** A validation study with four participating laboratories and a total set of 37 test chemicals comparing average lethal end points of the alternative test with data on adult zebrafish or, where not available, with acute data on the golden orfe, revealed a slope of 0.81 in the regression analysis and a regression coefficient of 0.87. The OECD adopted this method as a new guideline.

The test has already been used to assess the toxicity of wastewater (16). Further validation that includes an international inter-laboratory study with 11 laboratories is in progress. Results obtained using this test have also been compared with data from *Daphnia* acute immobilization test and RTS-2 cell tests. There were few cases where the *Daphnia* test was more sensitive. The RTS-2 cells are generally less sensitive

**Table 3.** Relative frequency of various types of alternatives to live vertebrates in scientific publications.<sup>a</sup>

	Document (journal article or report) publication date					
	1990 (n=80)	1991 (n=209)	1992 (n=563)	1993 (n=582)	1994 (n=493)	1995 (n=405)
Vertebrates						
Higher vertebrates, cell lines	12.5	8.7	9.7	10.8	8.9	8.4
Higher vertebrates, cell cultures	35.0	31.4	31.9	25.4	22.2	34.7
Higher vertebrates, embryos, organs, organ slices	15.0	14.0	14.5	12.9	11.5	10.8
Artificial organs	0	1.4	2.7	2.1	4.3	0.8
Lower vertebrates, cell lines	0	0	0	1.4	0.5	0.3
Lower vertebrates, cell cultures	0	1.0	0.9	0.9	0.7	1.0
Lower vertebrates, embryos, organs, organ slices	2.5	1.9	0.7	0.9	0.2	0.3
Invertebrates	6.25	1.0	0.7	0.5	1.4	1.0
Plants	1.25	0	0.4	1.0	0.2	1.0
Bacteria, fungi, protozoans	5.0	3.9	2.5	3.6	2.2	3.2
Methods	8.75	15.9	18.0	22.5	27.8	14.2
Reviews	11.25	11.1	11.5	9.1	9.1	12.4
Structure-activity relationships	0	4.3	0.5	1.0	1.2	1.6
Miscellaneous	2.5	5.4	6.0	7.9	9.8	10.3

n, total number of references identified for the year noted. <sup>a</sup>Data from the National Library of Medicine/U.S. National Institutes of Health (62).

(lg EC<sub>50</sub> RTS-2 = 0.69 lg LC<sub>50</sub> embryo + 1.47;  $r = 0.807$ ,  $n = 17$ ).

**Nondestructive Assays upon Vertebrates.** In vertebrates there has been considerable interest in the use of nondestructive biomarkers of toxic effect in laboratory and/or fetal studies. A number of assays of this type using blood samples have already shown promise (2,4). Included here are changes in blood-clotting proteins (anticoagulant rodenticides), changes in retinol and thyroxine (PCB metabolites), changes in porphyrin levels (organohalogen compounds), assays for DNA damage in white cells (carcinogens and mutagens), and vitellogenin production in male fish (environmental estrogens).

In laboratory and in field studies, inhibition of blood cholinesterase and carboxylesterases has provided indices of exposure to organophosphorus insecticides (17). However, such assays have provided only biomarkers of exposure, and there is no simple relationship to subsequent toxic effect. In birds, sublethal effects of pesticides on reproductive success have sometimes been investigated. Egg production and hatchability were end points used (18). In other studies in the laboratory and in the field, changes in the levels of porphyrins and retinol have been related to exposure to pollutants (19).

**Alternative Methods Already Developed for Individual Invertebrate Species.** SPECIFIC CONSIDERATIONS FOR INSECTS. At present insects are frequently being used in alternative testing methodologies due to specific characteristics which can be summarized as follows: *a*) insects show extremely high species diversity, with up to 1.5 million species already described; *b*) insects are very sensitive to some chemicals, especially to pesticides (usually much more sensitive than vertebrates); *c*) insects possess relatively high fecundity and short life cycles; *d*) standard rearing procedures have been elaborated for numerous species so that keeping insects is relatively simple, and almost unlimited numbers of individuals can be easily obtained at very low cost. Using insects is not generally restricted by law and there are only a few protected species (e.g., bees). The public largely will tolerate the experimentation with insects, unlike toxicity testing in mammals.

However, there are limitations to using insects in replacement for vertebrates in testing chemicals. For example, rigid chitinous integument and tracheal respiration are features of insects not found in vertebrates. Insect resistance to pesticides or

other chemicals must be taken into account. Within natural populations of many species, those genotypes are gradually selected whose makeup ensures survival even when the insects are treated with much higher doses than those killing susceptible populations. Numerous cases of cross-resistance have been described as well. The undesirable effects of resistance can be prevented by using susceptible strains. Unfortunately, strains that are guaranteed by the World Health Organization or the Food and Agriculture Organization are available only for some species (e.g., house flies, mosquitos, and some aphids).

There are also many differences in the action of chemicals between insects (invertebrates) and vertebrates. There are differences in uptake, pharmacokinetics, metabolism, and detoxification pathways. Some harmful effects of chemicals upon vertebrates cannot be tested in insects at all. For instance, it is very difficult to test carcinogenicity, as tumorlike proliferation in general is very rare in insects. On the other hand, insects seem to be suitable models for testing the effects of chemicals on reproduction. Our knowledge of insect reproductive systems is relatively extensive, especially of ovaries and fecundity. It is relatively simple to evaluate the histopathological changes of ovarian tissue. The high fecundity of most species enables rapid detection of the effects of chemicals on egg number, viability, and hatchability. Most invertebrates have very short embryogenesis and thus embryolytic effects can be easily studied.

However, the possibility of testing genotoxic effects in insects is somewhat restricted as there are only a few species for which the genetics are well known. In this respect, *Drosophila melanogaster* (and some other species of this genus) is an exception. The genome of this species is known in detail; short generation time, numerous progeny, few chromosomes, and numerous diverse markers enable detection of effects such as dominant lethal mutation, chromosome loss, deletions, translocations, nondysfunctions, and recombination. Several standard mutagenicity tests have been developed in *Drosophila*. Examples include sex-linked recessive lethal test, translocation assay, aneuploidy test, or urine-spot test.

TESTS DEVELOPED IN INVERTEBRATES. In situations where microorganisms, cultured cells and tissues, and other *in vitro* methods were unsuitable replacements for animals, invertebrate species have received particular attention (1). The use of horseshoe crab

(*Limulus polyphemus*) instead of rabbit for pyrogenicity testing constitutes perhaps the best example of such an alternative, as it has totally replaced the classical rabbit test. This test, based on the use of a lysate of *L. polyphemus* amoebocytes, is simpler, more rapid, and more sensitive than the corresponding vertebrate test (20).

**Developmental toxicity testing.** The use of invertebrates was the first alternative to classical tests in developmental toxicity testing. An *in vitro* teratogen assay has been developed that uses *Drosophila* embryo cell cultures (21). Various experiments have shown that this assay can be used as a teratogen screen; in mechanistic studies of abnormal development, it can be used to investigate gene involvement in teratogenic resistance, and the possible role of heat-shock proteins in preventing birth defects (21–24).

Two of the proposed developmental toxicity prescreen assay systems based on invertebrates use the coelenterate *Hydra attenuata*. The regeneration assay using body segments appears to be ineffective for the prescreening of chemicals for selective developmental toxicity hazard-potential; however, the use of the artificial embryo in the *Hydra* developmental toxicity assay agrees with published vertebrate studies (25–29).

**Genotoxicity testing.** A number of genotoxicity tests based on *D. melanogaster* have been proposed. These tests are of two types, detecting either somatic (somatic mutation and recombination test [SMART]) or germinal (sex-linked recessive lethal test [SLRLT]) mutations. SLRLT is the best-validated *Drosophila* assay but SMART protocols are less time consuming and can detect a broad range of genetic alterations using well-known genetic markers (eye color, wing cells, hairs, etc.). However, results frequently depend on the *Drosophila* strain used, especially for compounds that require metabolic activation (30).

Recently, the micronucleus test has been performed on marine mollusks to evaluate genotoxic effects of pollutants released in the marine environment (31). Mussels and earthworms also have recently been used to detect DNA single-strand breaks caused by contaminants of marine water and soil, respectively. For this purpose, the comet assay has been adapted to isolated cells (coelomocytes in earthworm and digestive gland cells in mussels).

**Invertebrate pharmacological models.** Selected organs, tissues, or cells of some invertebrates are being extensively used to elucidate mechanisms involved in drug and

environmental chemical toxicity. Among these, nervous structures (squid giant axon, crayfish giant axon, and stretch receptor organ, snail neurones, cockroach nerve cord and giant axon, and insect-isolated neurons) are frequently used to investigate the effects of neuroactive substances such as pesticides.

**Carcinogenicity.** Even though tumorlike lesions have sometimes been reported in wild invertebrates (mainly shellfish), a clear link with mutagen/carcinogen concentrations in tissues has rarely been established. There is no evidence that these invertebrates may constitute a valuable model of mammalian carcinogenesis.

More attention should be devoted to field studies, as much of the literature pertaining to the toxic effects of chemicals is based on laboratory observations and it is difficult to extrapolate the data to invertebrates in nature.

### Structure-Activity Relationships

The principle of QSAR is highly applied in the field of development of bioactive compounds, particularly in the agricultural and pharmaceutical fields. There are two major aspects: one is the identification of new basic structures, the other is the optimization of the particular effect devised for the new substance. In ecotoxicology, QSAR is used to estimate potential effects of existing chemicals on certain organisms or end points. For the majority of such, there is no a priori knowledge of which organism/genera is most sensitive, the mode of action, distribution/partitioning within the ecosystem, metabolic breakdown products, and so forth.

**Partitioning within Ecosystems.** Much progress has been achieved by using physico-chemical parameters, notably measures of lipophilicity (i.e., the octanol/water partition coefficient and volatility from aqueous solutions, vapor pressure, Henry coefficient) to model the distribution of a chemical between major ecosystem compartments. Several quantitative models have been developed, with the fugacity model of Campfens and Mackay (32) and Severinsen et al. (33) among the best known. Both the computation of many of the physicochemical properties as well as distribution/fate models are well advanced.

**Metabolic Breakdown.** There are several artificial intelligence/knowledge-based systems and related algorithms that allow the computation of metabolic pathways and intermediates. Although far from perfect, these models give indications of

potential enzyme blockers or activators as they may arise within an organism.

**Special Considerations.** OZONE-DEPLETING SUBSTANCES. These materials are highly volatile, low molecular weight halocarbons whose physicochemical properties (volatility and resistance to biological/abiological breakdown) makes them ascend to the upper atmosphere where they become photochemically activated and effect a catalytically destroy ozone. The chemical process is well documented and most compounds of concern are directly produced or emitted. In principle, however, some could also be derived from the chemical breakdown of large molecules. QSAR models on the activity/persistence of ozone-depleting substances are available (34).

GROUNDWATER. At present, no validated methods exist to test for biological effects of organic chemicals in aquifers. Hence, the criteria for risk assessment of groundwater are degradability, sorption, and accumulation only. For groundwater risk assessment of pesticides, interpretive field studies, lysimetric methods, and validated mathematical models (e.g., pesticide root zone model [PRZM], pesticide eaching model [PELMO]) are in regulatory use and run under Good Laboratory Practices.

EFFECTS. QSAR is most widely used for the estimation of toxic effects of chemicals. Models for the narcotic effects have been developed for fish (35,36) for the effects of nonpolar (e.g., hydrophobic) and polar narcotics (e.g., phenols). For compounds of certain chemical groups, phenols, anilines and so forth, good correlations are often found with the octanol/water partition coefficient ( $\log P$  or  $\log K_{ow}$ ). Chemicals with several functional groups are more difficult to predict. Specific activity (change in mode of action) can occur in simple series (e.g., chlorophenols) where lower chlorinated compounds are narcotics and higher chlorinated ones are blockers of oxidative phosphorylation. Similar changes in mode of action have been noted in the phenol, nitrophenol, 2,4-(NO<sub>2</sub>)-phenol series.

Photochemical activation of polycyclic aromatic hydrocarbons within organisms results in highly toxic compounds. Correlation of parent molecule toxicity with molecular energy levels has been proposed and demonstrated (37).

Toxicity of lipophilic compounds (e.g., PCBs) depends on stereochemical/optical isomerism/flexibility (38,39), as determined by *ortho*-substitution. Toxic equivalents of individual isomers/congeners

can be correlated with such structural features/properties (40).

**Interspecies Correlations.** AQUATIC SPECIES. The existence of large published datasets of toxicity measurements for single species allows quantitative interspecies comparisons. These datasets include approximately 1600 chemicals on photobacterium (11), approximately 800 chemicals on fathead minnow (*Pimephales promelas*), and approximately 400 chemicals on *Tetrahymena pyriformis* (41). They allow the development of both intraspecies QSAR and interspecies comparisons. For the latter, several publications demonstrate the possibility of quantitatively predicting effects between related and nonrelated species (42). In general, these results are very encouraging and can be applied to acute, subacute, and chronic end points for many genera and species in the aquatic sphere. A table of interspecies correlations of a variety of subsets of chemicals is given in Kaiser (3). For a holistic approach that disregards type/class of molecules or mode of action for up to several hundred compounds, highly significant correlations between *V. fischeri* and fathead minnow as well as many cold and warm water freshwater fish species (including rainbow trout, zebrafish, golden orfe, flagfish, catfish, carp, goldfish, bleak), and marine fish (sheepshead minnow) have been demonstrated. Similar relationships exist for a variety of algae (*Scenedesmus*, *Chlorella*), ciliate (*Tetrahymena*), and other organisms (*Daphnia*, *Nitrocras* spp.). Large crustaceans (*Crangon* sp., *Artemia* sp.) are frequently very sensitive to certain types of compounds and interspecies models are not yet fully developed. For mechanistically defined groups of chemicals (e.g., alcohols, phenols), interspecies correlations between *Vibrio* bacteria and fish are highly significant. This observation extends to complex chemicals also, but certain features/groups (e.g., vinyl derivatives) have higher fish toxicity than expected from the bacteria. Once known, such effects can be compensated for and, at the very least, allow the identification of those compounds for which such correlations should be viewed with caution.

TERRESTRIAL SPECIES. Interspecies correlations between *Vibrio* data and toxicities to plants, insects, and mammalian species have been investigated in several papers. By far the largest number of chemicals (>500) investigated are rat and mouse acute toxicity data. A table of correlations between earthworm (*Eisenia foetida*) and rat is given in Lagadic and Caquet (1). Of



these, the best correlations were obtained for intravenous exposure LD<sub>50</sub> values, as these are the least influenced by metabolic and kinetic effects. The resultant correlations allow order-of-magnitude estimations from these interspecies toxicity relationships (42).

**Developments and Outlook.** Recent developments in the field of artificial intelligence that employ various neural net-type algorithms have brought a new impetus to the field (43–45). Improvements can be expected in several directions.

**Comments regarding Data Quality.** A note should be made in regard to data quality. In the development of new pesticides, and drugs, the QSAR analysis can normally rely on experimental data for which the mean variation may be 0.1 to 0.2 log units. In the aquatic field, particularly for interspecies comparisons, the data quality would frequently be much less, particularly where static tests (*Daphnia*, algae, fish) are used vis-a-vis flow-through tests (96-hr fathead minnow, rainbow trout). In most cases, concentrations are nominal and the effects of volatilization, degradation, and absorption in static systems are not quantified. For interspecies comparisons of ionizing substances, pH control/knowledge is also of major importance but often not available. In combination, these variations can lead to apparent discrepancies and differences in sensitivities that in reality are based on experimental conditions rather than on inherent differences in species sensitivity.

### Mesocosms

The value of mesocosms is mainly based on the combination of ecological realism, achieved by introduction of the basic components of natural ecosystems, and facilitated access to a number of physicochemical, biological, and toxicological parameters that can be controlled to some extent. Mesocosm structure and use have been described in detail in recent publications (46,47). Aquatic mesocosms are sometimes required for the registration of new chemicals, especially pesticides (48–50). Readers can refer to recent guidelines proposed by the OECD (51) and the U.S. Environmental Protection Agency (U.S. EPA) (52) for the use of aquatic mesocosms for regulatory purposes. Chemical testing in mesocosms is more realistic than laboratory tests and easier than field assessment of chemical effects. Therefore, mesocosms are often considered an experimental situation between laboratory testing and field evaluation. In this respect the more suitable approach for

using mesocosms in ecotoxicological risk assessment consists in a couplage with standardized laboratory tests.

In such a context, how does ecotoxicity testing in mesocosms meet the essential concern of alternative methodologies summarized by the Russell and Burch's 3Rs (53).

**Refinement:** Certainly, the use of mesocosms refines the classical methods of ecotoxicological risk assessment as they provide conditions for a better understanding of environmentally relevant effects of chemicals. Indeed, mesocosms provide a more realistic approach for the evaluation of effects of chemicals at many different levels of organization (from the molecule to the population and community), for different types of organisms, from bacteria to invertebrates and lower vertebrates. They also appear to be potent tools for predicting changes at the highest levels of organization (population, community, and ecosystem) from measurements of individual end points.

**Replacement:** Ecotoxicological investigations in mesocosms do not entirely replace the use of animals. However, they allow the tests to be performed on species that are not of major societal concern, but which play key roles in the structure and functioning of ecosystems. For example, investigations of chemical effects in freshwater mesocosms have largely used invertebrate species because of their importance in aquatic food webs (1).

**Reduction:** To some extent, investigations of ecotoxicological effects in mesocosms can dramatically reduce the need for animals when, in a particular test, ecosystem-relevant functional end points can be measured. Among those end points, plankton respiration, phytoplankton photosynthesis, concentrations of chlorophyll *a*, dissolved oxygen or nitrogen, and ammonium are the most commonly measured in aquatic ecosystems.

The need for using animals in ecotoxicity testing in mesocosms clearly depends on the end points that must be assessed. In this respect, mesocosms allow nondestructive measurements of integrated end points (endpoints at high levels of organization or functional end points). Chemical tests in mesocosms should therefore be designed to reduce or even replace the use of vertebrates, or to reduce the amount of suffering of vertebrates by measuring nondestructive parameters.

### Community/Ecosystem Studies

The study of the exposure and effects of environmental contaminants at the

community and ecosystem levels of ecological organization is critical to our understanding of overall environmental health and potential impacts on plant and animal species as well as humans. These impacts can be direct effects or can be more subtle indirect effects, whereby structural or functional components of the ecosystem are altered, leading to subsequent impacts on other interrelated components. Community and ecosystem studies fall into two distinct categories: *a*) monitoring of communities and ecosystems, and *b*) controlled experimentation. Monitoring communities and ecosystems can include such types of studies as food chain/food web studies, structural and functional analyses, and indices of biotic integrity (e.g., IBI's in the United States, RIVPAC's in the United Kingdom).

Food chain/food web studies are efficient methods of exploring exposure and effects of environmental contaminants in the environment. Partial or complete food chains, such as the zooplankton–fish–hawk food chain, have been examined for assessment of bioaccumulation and effects of environmental contaminants. Effects from food chain/food web studies can be assessed through use of a biomarker strategy (4). Again, a vast majority of these studies have been conducted in aquatic systems. Structural analyses of communities and ecosystems have been completed investigating such ecological parameters as species richness and abundance, comparative mean densities, and presence or absence of certain indicator (sensitive or tolerant/resistant) species. Functional analyses of communities and ecosystems include the examination of niche metrics (e.g., niche breadth, width), presence or absence of certain functional niches (e.g., decomposers, carnivores), and critical ecosystem functions, such as decomposition, energy flow, nutrient cycling, succession. To date, little ecosystem functional analysis has been completed. Indices of biotic integrity are a special subset of structural and functional analysis and are quantitative indices of community and ecosystem structural and functional attributes that potentially can be used in a regulatory framework for environmental health. These indices have been used exclusively in aquatic environments; work on indices of biotic integrity in terrestrial environments is badly needed. Controlled experimentation studies of community and ecosystem structure or function involve the use of microcosms, mesocosms, and other similar experimental designs. These experimental systems allow control of certain parameters

(e.g., selection and densities of test species, movements of and predation on test species) and simultaneous evaluation of multiple ecological levels that can be crucial to a better understanding of exposure and effects at all levels of ecological organization from the individual to the ecosystem.

## Future Developments

### Selection of Species. Problems of Extrapolation between Species

In selecting species for testing in the context of environmental risk assessment, there is the problem of choosing just a few surrogates for the extremely large number of species exposed to pollutants in the natural environment. There are very large differences between species in susceptibility to chemicals that must be considered. For this reason large safety factors are used when estimating environmental toxicity from laboratory toxicity data during the course of environmental risk assessment. A general increase in the number and range of test species will provide no practical solution to this dilemma. With the growth of knowledge and new technologies (see later discussion), it is expected that there will also be some changes in species, strains, and development stages used in testing procedures. These will represent more appropriate choices for particular chemicals than certain species used in present testing procedures. Such changes can also benefit our current interest in alternative methods that will follow the principles defined by the 3 R's. One change we may expect is the use of transvected organisms that can be tested for particular mechanisms of toxic action, as these become available with advances in biochemical toxicology. Also, a more ecological approach will draw attention to particular life stages that may be especially vulnerable to the effects of specific pollutants. There is continuing concern over the differences in susceptibility between wild species and laboratory strains, even when they are of the same, or closely related species (54). Invertebrates differ from vertebrates in many respects and cannot sensibly be regarded as surrogates for them in toxicity testing. The development of non-destructive assays for vertebrates, in the laboratory or in the field, may provide a way of overcoming the problem.

It is evident that the problem of making species comparisons in ecotoxicology is much more complex than it is in classical toxicology where all comparisons are being made with a single species. The extent to

which strategies to assess the harmful effects of pollutants on natural populations (e.g., by the use of biomarkers) may be beneficial in the context of human health assessment is not easy to judge.

### Refinement of Nondestructive Vertebrate Assays

A central concern of the present report is reduction of harmful testing of vertebrates. The most straightforward approach to this problem, at least in the short term, is to make greater use of nondestructive assays upon vertebrates, which can be applied in the laboratory, and more importantly, in the field. The promise of this line of approach has been discussed elsewhere (2,4). Thus molecular and cellular responses to pollutants can be measured in blood, skin, eggs and feces. Nonlethal biochemical, physiological, immunological, and behavioral effects of pollutants can also be measured under field conditions (2).

### New End Points

The ever-increasing knowledge from molecular biology, transgenic organisms, and aspects of clinical testing provide a rich resource for ecotoxicologists in devising new tests. Here we present a brief discussion of new end points that we consider to have potential in the field of ecotoxicology.

**Genotoxicity.** A number of methods are in existence for measuring DNA damage, and the use of DNA adduct formation in several aquatic species to demonstrate environmental exposure is well established. However, controversy exists in the scientific community as to the wider relevance of these changes. There are arguments that genotoxicity impacts the gene pool and therefore has significance in environmental assessment. The other major argument is that mutants will not be viable and therefore are nonrelevant. This is likely to remain an important issue until there is a better understanding of the environmental relevance (or otherwise) of genotoxicity.

One approach that has attracted considerable interest recently is the DNA fingerprinting technique incorporating the use of polymerase chain reaction (PCR). There are reasons for believing that this can provide evidence for the presence of specific DNA adducts as well as mutations (55). An assay such as randomly amplified polymorphic DNA can also provide evidence of reduction of genetic variability caused by pollutants, an effect that could have important evolutionary implications for exposed organisms (56).

**Carcinogenicity.** Information from human carcinogenicity testing using available methods should be the basis of considering the possible applicability to fish. A confirmatory methodology, either environmental surveillance or experimental testing, should be used on a case-by-case basis.

**Cell Culture Systems.** It has been well established that cultured cell lines are inappropriate as quantitative substitutes for *in vivo* systems at the present state of knowledge. Primary cells do not have this disadvantage, but are limited in use, considering the kinetics of *in vivo* systems. An important use of cell cultures, however, is in qualitative recognition of mechanisms. For this purpose, extensive validation and further standardization of cell culture systems will improve information for assessment.

Many new possibilities exist using cell lines for species that are difficult or impossible to study in the lab. The production of transvected cell lines may increase the usefulness of this approach.

**Olfactory Responses.** Olfactory responses such as changes in breathing rhythm, cough/gill purge frequencies, and cough amplitude of fish have been used repeatedly to analyze the effects of exposure to low levels of chemicals in water. Recent developments in computerized analysis of patterns (57) have resulted in commercially available systems allowing fast, online monitoring of water supplies. Furthermore, research tests with several types of xenobiotics have indicated high sensitivity of such systems and potential differentiation in the response for different chemicals such as narcotics and membrane irritants (58).

**Endocrine Effects.** Field observations indicate that endocrine effects in the environment could be an important end point of ecological relevance. A considerable number of candidate chemicals of varying degrees of potency, and a number of widely differing responses have been reported. Frequently used methods include estrogen receptor assay, vitellogenin synthesis in egg-laying vertebrates, proliferation of human tumor cells, aromatase assay, changes in gonad and gamete structure and function, imposex induction in whelks, and proliferation of the kidney in sticklebacks. Fish life-cycle testing has been proposed as the gold standard for a broader range of endocrine end points. Despite substantial efforts, three crucial questions need to be solved:

- The hazard posed by endocrine chemicals at the population level



- Validation and standardization of methods in comparative studies
- The contributions (if any) and synergistic actions of naturally occurring phytoestrogens in the endocrine-disruption picture.

### How Can Mesocosms Improve the Measurement of Classical End Points?

Classical end points can be measured at any level of biological organization of the mesocosm. Responses measured at the level of the individual and below refer to toxicological end points (biomarkers). As such, those parameters can benefit from alternative approaches proposed for higher vertebrates (as has also been discussed in other working groups). Parameters measured at the levels of population, community and ecosystem refer to ecological end points. They include both structural and functional end points and are mostly nondestructive for vertebrates. The main advantages of mesocosms for the assessment of these end points can be listed as follows:

- Mesocosms allow kinetic studies of the fate of chemicals. At the individual level, kinetic parameters describe distribution of chemicals among target and nontarget tissues. Similarly, kinetic approaches could be developed in mesocosms to describe (and to model) the transfer of chemicals between the different compartments (both physical and biological).
- Tests on various life-stages (including eggs) in realistic conditions of exposure can be performed using mesocosms.
- Mesocosms allow a sound evaluation of responses that have no ecotoxicological meaning (or cannot simply be measured) in the laboratory and for which measurements are hardly replicable in the field. This especially concerns population/community structure and functional parameters (pH, nitrogen cycle, photosynthesis/chlorophyll *a*, recycling of organic matter, and adenosine triphosphate [ATP], community respiration, etc).
- In mesocosms, both individual and ecological responses to chemicals are more easily interpretable because of the possibility of establishing dose-response relations. Such an opportunity is important, for example, in the procedure of evaluation and validation of biomarkers prior to their field use.
- Simultaneous measurements of parameters in both treated and control mesocosms allow discrimination between

natural variations and chemical-induced changes for both individual and ecological end points. The use of real-time controls is of great value for the validation of biomarkers.

- Individual and ecological responses to chemicals can be followed over long periods of time (kinetic approach). To some extent, mesocosms allow a good repeatability of nondestructive procedures (sampling on the same individuals).
- In mesocosms, methods that are going to be used in natural ecosystems can be improved, especially in order to reduce the need for field-collected animals. Mesocosms also offer suitable conditions for cross-reference studies between species in order to identify replacement species.
- Mesocosms provide conditions for the identification and study of mechanistic links between individual end points and ecological end points. In other words, mesocosms facilitate extrapolation across levels of biological organization. This is the experimental basis for the use of biomarkers as predictive indicators of effects at higher levels of biological organization (population and community).

### Extrapolation between Organizational Levels

Testing at higher levels of organization using microcosms, mesocosms, or in the field generally results in reduced possibility of demonstration of individual effects, as not all species present are investigated and the affected ones can be substituted by others with regard to their function. This limits the use of functional parameters and argues in favor of single-species testing. Future methodological developments therefore should emphasize selection and relevance of indicator species for the parameters measured in testing at higher levels of ecological organization.

Indices based on presence/absence and abundance of invertebrate species have largely been developed through animal bioindication approaches and address responses at the community level. However, they give only an instantaneous picture of the state of an ecosystem, based on changes in species diversity and richness as ecological conditions have changed. In particular, such nonspecific, macroscopic parameters fail to reveal contamination of individuals and subsequent biochemical or physiological changes that may affect maintenance, growth, and reproduction. Individual contamination and biochemical/physiological

changes can be assessed through chemical analysis and biomarker measurements, respectively. From this point of view, invertebrates do not differ from vertebrates, and it can reasonably be stated that any of those measures can be conducted equally in individuals of both groups (1).

Recent investigations have clearly highlighted the interest of using invertebrates to link individual responses with changes in populations or communities, as such correlations will be of great value in rapid, early-warning assessment of the environmental impact of chemicals. The use of invertebrates in such a strategy may prevent adverse effects occurring in vertebrates, and eventually in humans. To link, in a mechanistic way, individual responses assessed through biomarker measurements to changes at population and community levels is probably one of the most important initial steps for the definition of early-warning indicators of environmental impact of chemicals. Invertebrate species have proved to be particularly suitable for such investigations, as organismal changes can rapidly affect the population (59,60). This is not the case for fish or mammal species traditionally used in biomonitoring programs. Recent studies of the effect of endocrine disruptors on aquatic invertebrates have provided evidence to support the existence of causal links between organismal responses and changes at population or community levels (1). Mechanistic linkage between effects at different levels of biological organization has also been achieved using the freshwater amphipod *Gammarus pulex* in which changes in physiological energetics have been linked to community function that may be indicative for changes in community structure (60).

### Ecological End Points

In ecotoxicology, the ultimate concern is with effects at the level of population community and ecosystems. The difficulty of extrapolating from toxic effects upon individuals to effects at these higher levels of organization has already been stressed. There is, however, the possibility of taking a top-down approach, seeking to measure effects at these higher levels of organization, as in the case of effects upon ecosystem function previously described. Other effects that may receive more attention are upon population dynamics and population genetics.

**Population Dynamics.** In population dynamics, ecotoxicologists are concerned with the relationship between energy costs borne by organisms in mounting defense

systems (e.g., induction of detoxication enzymes, and metallothionein) and in Darwinian fitness. Species heavily impacted by pollutants may decline, because they have too little energy to expend on reproduction and growth. Assays of scope for growth can provide a measure of this surplus energy (61). Such considerations are not given much attention in chemical toxicology, where the concern is with toxic mechanisms in individuals.

**Development of Resistance.** The potential of certain species to develop resistance upon exposure to chemicals, resulting in the emergence of resistant strains, is an effect that can provide a measure of the environmental impact of chemicals. Methodology can be developed based on experience with pesticide resistance. (The

development of resistance in certain species, e.g., of insects, leads to the emergence of resistant strains.) The identification of resistant strains (e.g., by toxicity tests, immunochemical assays, or DNA probes) can provide evidence for the selective environmental impact of particular chemicals in defined habitats (e.g., aquatic organisms in polluted waters; soil organisms near mine workings with high  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ , etc.).

## Recommendations

- There is a need to more clearly define strategies and goals when undertaking testing procedures.
- In the context of environmental risk assessment, the objectives of the 3Rs will be served by *a*) developments and improvements in assays incorporating

new techniques from biochemical/molecular biology that relate to mechanisms; *b*) further development of nondestructive assays for vertebrates, and assays for invertebrates; *c*) selection of the most appropriate species, strains and developmental stages in the light of new knowledge (but no additional vertebrate species for basic testing); and *d*) better integrated approaches incorporating biomarker assays, ecophysiological concepts, and ecological end points. Maximum success depends on a flexible approach and expert judgment in interpretation.

- Testing protocols need to be realistic, taking into account particular problems with mixtures and volatile or insoluble chemicals.

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